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Antagonistic actions of Msx1 and Osr2 Pattern Mammalian Teeth into Single Row**

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Abstract

Mammals have single-rowed dentitions whereas many non-mammalian vertebrates have teeth in multiple rows. Neither the molecular mechanism regulating iterative tooth initiation nor that restricting mammalian tooth development in one row is known. We found that mice lacking the transcription factor Osr2 develop supernumerary teeth lingual to their molars due to expansion of the odontogenic field. Osr2 was expressed in a lingual-to-buccal gradient and restricted expression of Bmp4, an essential odontogenic signal, in the developing tooth mesenchyme. Expansion of odontogenic field in *Osr2*-deficient mice required Msx1, a feedback activator of *Bmp4* expression. These findings suggest that the Bmp4-Msx1 pathway propagates mesenchymal activation for sequential tooth induction and that spatial modulation of this pathway provides a potentially general mechanism patterning vertebrate dentition.

Teeth are vertebrate-specific organs and distinct dentition patterns have played critical roles in vertebrate diversification and specialization (1–3). In addition to variations in tooth number, size, and shape, many non-mammalian vertebrates have multi-rowed dentitions whereas mammals develop teeth in a single row. Studies of tooth development in several fish species showed that multi-rowed dentitions result from sequential iterative tooth initiation along the mesial-to-distal and labial-to-lingual directions (3–5). The molecular mechanisms regulating the precise spatiotemporal patterns of sequential tooth initiation are unknown. Development of the single-rowed mammalian dentition likely involves restricting odontogenic field along the buccolingual axis; the mechanism underlying this control is also unknown.

Current understanding of the molecular mechanisms controlling tooth development has come mostly from studies in mice (1,6). Although supernumerary teeth have been reported in several mutant mouse strains (7–10), the majority developed within the tooth row from vestigial diastemal tooth buds (10,11). We recently generated mice lacking the *Osr2* (*odd-skipped related-2*) gene (12,13) and found that they exhibited supernumerary tooth development lingual to their molar teeth (Fig. 1A, B, and Fig. S1). Histological analyses (14) traced initiation of these supernumerary tooth germs to aberrant thickening of oral epithelium lingual to the first molar tooth buds at E13.5 (Fig. 1C, D). By E15.5, as the first molar germs developed to late "cap" stage (1), the ectopically thickened oral epithelia in *Osr2*$^{-/-}$ embryos invaginated and

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the underlying mesenchyme condensed (Fig. 1E). These ectopic epithelial invaginations resembled cap stage tooth germs by E16.5 (Fig. 1F). Because *Osr2*$^{-/-}$ mice die shortly after birth due to cleft palate (13), we transplanted E13.5 mandibular molar tooth germs under renal capsules of adult mice to allow complete tooth morphogenesis (14). After 21 days, wildtype and heterozygous molar tooth germs gave rise to two to three mineralized molar teeth, representing the normal molars (Fig. 1G). In contrast, *Osr2*$^{-/-}$ mutant molar tooth germs gave rise to four to five mineralized teeth (Fig. 1H, and Fig. S2). These data indicate that a complete odontogenic program is ectopically activated lingual to the molar teeth in *Osr2*$^{-/-}$ mice.

To gain insight into supernumerary tooth formation in *Osr2*$^{-/-}$ mice, we examined expression of selected marker genes during early tooth development. *Pitx2* was initially expressed throughout oral epithelium and its expression selectively maintained in dental epithelium after E11 (15). In *Osr2*$^{-/-}$ embryos, *Pitx2* expression abnormally persisted in oral epithelium lingual to the first molar tooth buds (Fig. S3A, B). By E14.5, strong *Pitx2* expression marked the supernumerary dental placodes and first molar tooth buds (Fig. S3C, D). At E13.5, *Shh* was expressed in the enamel knot of developing molar tooth buds (16) (Fig. S3E). In *Osr2*$^{-/-}$ mutants, *Shh* was ectopically expressed in a subset of epithelial cells lingual to the first molar buds (Fig. S3F). By E14.5, *Shh* expression was clearly detected in the supernumerary dental placodes in *Osr2*$^{-/-}$ embryos (Fig. S3H). In addition, expression of dental mesenchyme markers *Msx1* and *Lef1* were upregulated and expanded lingually in *Osr2*$^{-/-}$ mice (Fig. S3, I–L). These data suggest that supernumerary tooth development resulted from lingual expansion of the odontogenic field from the first molar tooth germs.

To understand how Osr2 regulates the odontogenic field, we examined *Osr2* expression during normal tooth development. At E11.5, *Osr2* was strongly expressed in the mesenchyme lingual to the dental lamina in both the maxilla and mandible (Fig. 2A). *Osr2* was also highly expressed in the proximal mandibular mesenchyme buccal to the dental lamina (Fig. 2A). As tooth buds developed from E12.5 to E14.5, *Osr2* mRNA was expressed in a gradient in the developing tooth mesenchyme, with higher levels lingual and lower levels immediately buccal to the tooth buds (Fig. 2, B–D). Overall, the *Osr2* expression pattern is complementary to that of Bmp4 (Fig. 2, E–H), an essential odontogenic signal preferentially expressed on the buccal side in developing molar mesenchyme (17–20).

The expression pattern and mutant phenotype suggest that Osr2 functions to restrict odontogenic potential in the developing tooth mesenchyme. Consistent with this hypothesis, *Bmp4* expression is upregulated and expanded into mesenchyme lingual to first molar buds in *Osr2*$^{-/-}$ embryos by E13.5, compared with wildtype littermates (Fig. 3A, B, Fig. S4). Moreover, Smad1 activation was enhanced in the molar tooth germ and expanded lingually to the oral epithelium and mesenchyme in *Osr2*$^{-/-}$ embryos, compared with wildtype littermates (Fig. 3C, D).

To test whether mesenchymal odontogenic field is expanded in *Osr2*$^{-/-}$ mice, we examined the ability of isolated E13.5 mandibular mesenchyme to induce tooth formation in non-dental epithelia from E10.5 second branchial arches (14). The molar tooth mesenchyme from both wildtype and *Osr2*$^{-/-}$ embryos induced tooth germ-like structures in non-dental epithelia in recombinant explant cultures (Fig. 3E, F). While mandibular mesenchyme lingual to wildtype molar tooth germs had no odontogenic activity (Fig. 3G), mandibular mesenchyme lingual to *Osr2*$^{-/-}$ molar tooth germs induced tooth-characteristic changes in non-dental epithelia (Fig. 3H). We next cultured the recombinant tissues *in vitro* followed by transplanting them under renal capsules to allow complete tooth morphogenesis (14). Histological analyses showed that the mutant, but not wildtype, mandibular mesenchyme lingual to molar tooth germs induced tooth morphogenesis in non-dental epithelium (Fig. 3, E–H), confirming that supernumerary

teeth in *Osr2*$^{-/-}$ mice were induced by lingually expanded mesenchymal signals from the molar tooth germs.

We next investigated whether exogenous Bmp4 was sufficient to induce supernumerary tooth formation lingual to the developing first molars. Implantation of Bmp4-soaked beads lingual to the first molar tooth germs of E13.5 wildtype embryos did not cause supernumerary tooth initiation in any of eighteen mandibular explants examined (Fig. S5), suggesting that supernumerary tooth induction in *Osr2*$^{-/-}$ mutants involved activation of additional mesenchymal odontogenic signals on the lingual side of the first molars.

Activation of odontogenic potential, including *Bmp4* expression, in the dental mesenchyme is mediated by the transcription factor Msx1 (17–20). Mice lacking *Msx1* exhibited loss of *Bmp4* expression in the dental mesenchyme and molar developmental arrest at the bud stage (17,21). Since *Msx1* is expressed throughout the early tooth mesenchyme (20), the spatially restricted *Bmp4* expression in normal dental mesenchyme and the expansion of *Bmp4* expression in *Osr2*$^{-/-}$ tooth mesenchyme suggest that Osr2 repressed Msx1-mediated activation of odontogenic signals. To test this hypothesis, we examined tooth development in mice carrying mutations in both *Osr2* and *Msx1*. In contrast to early tooth developmental arrest in *Msx1*$^{-/-}$ mice, all five *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutant pups harvested at E18.5 showed first molar development to late bell stage, with well-patterned ameloblast and odontoblast differentiation (Fig. 4, A–C, and Fig. S6). While *Bmp4* expression was down-regulated in *Msx1*$^{-/-}$ first molar mesenchyme from E12 to E13.5, (Fig. 4D, E, and Fig. S4), it was partially restored in *Msx1*$^{-/-}$*Osr2*$^{-/-}$ littermates (Fig. 4F). By E14.5, both wildtype and *Msx1*$^{-/-}$*Osr2*$^{-/-}$ first molar germs had developed to the cap stage, with similar patterns of *Bmp4* expression (Fig. 4G, I) while *Msx1*$^{-/-}$ first molar germs remained at the bud stage with little *Bmp4* expression (Fig. 4H). By E15, both wildtype and *Msx1*$^{-/-}$*Osr2*$^{-/-}$ first molar germs had progressed to late cap stage and strongly expressed *Lef1* (Fig. 4J, L), a downstream target of Bmp4 signaling (17), while *Msx1*$^{-/-}$ first molar germs remained arrested, with little *Lef1* expression (Fig. 4K). At all stages examined, however, no supernumerary tooth development was detected in *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutants. Moreover, the pattern of *Osr2* expression during molar tooth development was similar in *Msx1*$^{-/-}$ and wildtype littermates (Fig. S7). These data indicate that Msx1 and Osr2 act antagonistically to pattern the tooth morphogenetic field by controlling the levels and spatial distribution of mesenchymal odontogenic signals along the buccolingual axis. Disruption of the balance of this antagonistic interaction may underlie supernumerary tooth formation, such as in *Osr2*$^{-/-}$ mice, and tooth agenesis, such as in *Msx1*$^{-/-}$ mice and in humans with *MSX1* mutations (21,22).

In *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutant mice, sufficient levels of Bmp4 were expressed to drive morphogenesis of the first molar teeth but no supernumerary tooth initiated. Remarkably, mandibular second molars also failed to develop in *Msx1*$^{-/-}$*Osr2*$^{-/-}$ mutant mice (Fig. S8). Kavanagh *et al.* (23) recently showed that mouse mandibular first molar tooth germ inhibited second molar development and proposed an inhibitory cascade model, in which initiation of posterior molars depended on a balance between intermolar inhibition and mesenchymal activation, to account for sequential molar initiation in mammals. Similar activator-inhibitor mechanisms have been proposed for periodic dentition patterning in other vertebrates (3–5, 24), but the molecular underpinnings have not been identified. Bmp4 and Msx1 have been shown to regulate each other in a positive feedback loop in the dental mesenchyme (17–19). Bmp4 also induced expression of ectodin, a secreted Bmp inhibitor whose inactivation caused fusion of first and second molars as well as extra teeth in mice (9,25). Taken together, these and our finding that Msx1 is required for expansion of the odontogenic field in *Osr2*$^{-/-}$ mice suggest that the Bmp4-Msx1 pathway is a driving force in the activator-inhibitor network regulating sequential tooth initiation. In mammals, Osr2 suppresses this pathway along the buccolingual axis to restrict molar development to one tooth row. Diversity in dentition patterns

in other vertebrates is likely due, at least in part, to evolutionary changes in antagonistic interactions regulating this pathway across the tooth morphogenetic field. In addition, reiterative initiation of other ectodermal organs, such as feather buds and taste papillae, which are also controlled by epithelial-mesenchymal interactions (6,25,26), may be driven by propagation of mesenchymal activators through a similar mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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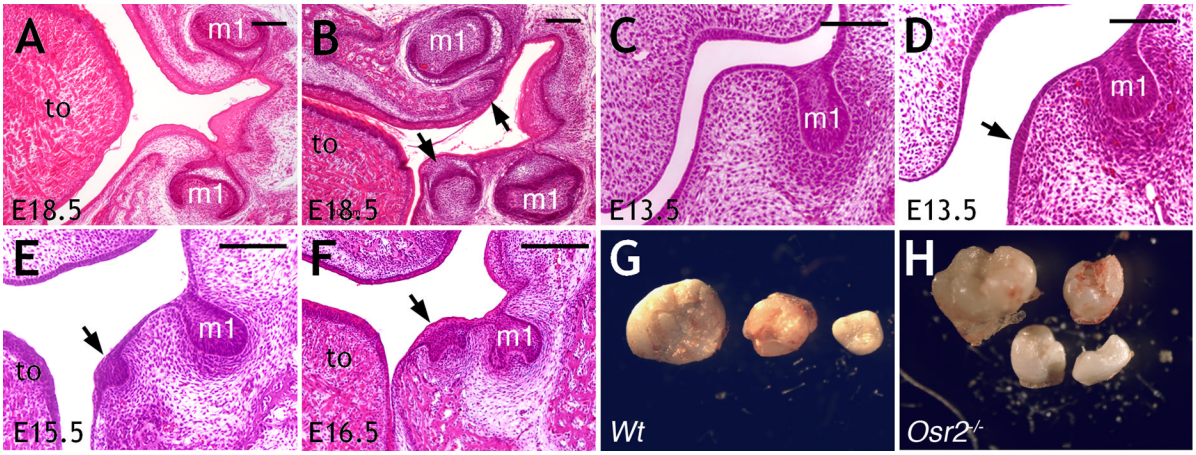

Fig. 1.

(**A, B**) Frontal sections of E18.5 wildtype (A) and *Osr2*$^{-/-}$ (B) littermates. Arrows in B point to supernumerary tooth germs. (**C**) Frontal section of wildtype first molar region at E13.5. (**D–F**) Frontal sections of *Osr2*$^{-/-}$ mutant first molar regions at E13.5, E15.5 and E16.5. Arrows point to supernumerary tooth germs. (**G, H**) Mineralized teeth from renal capsule cultures of E13.5 wildtype (G) and *Osr2*$^{-/-}$ (H) molar tooth germs. m1, first molar tooth germ; to, tongue. Scale bar, 100 μm.

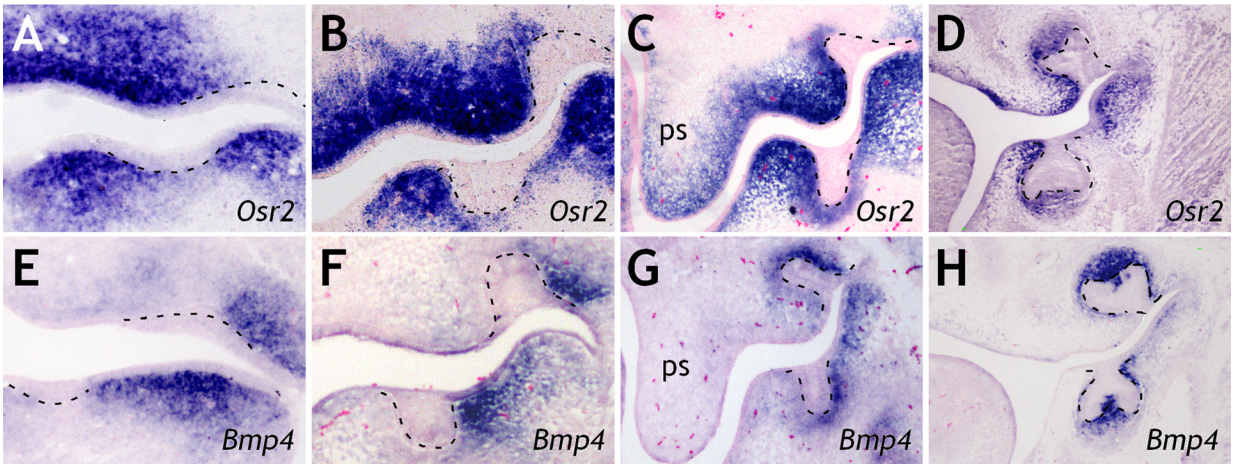

Fig. 2.

Expression patterns of *Osr2* (**A–D**) and *Bmp4* (**E–H**) mRNAs along the buccolingual axis of mouse molar tooth germs at E11.5 (A, E), E12.5 (B, F), E13.5 (C, G), and E14.5 (D, H). Lingual side is to the left in all panels. Black dashed lines mark the boundary between dental epithelium and mesenchyme. ps, palatal shelf.

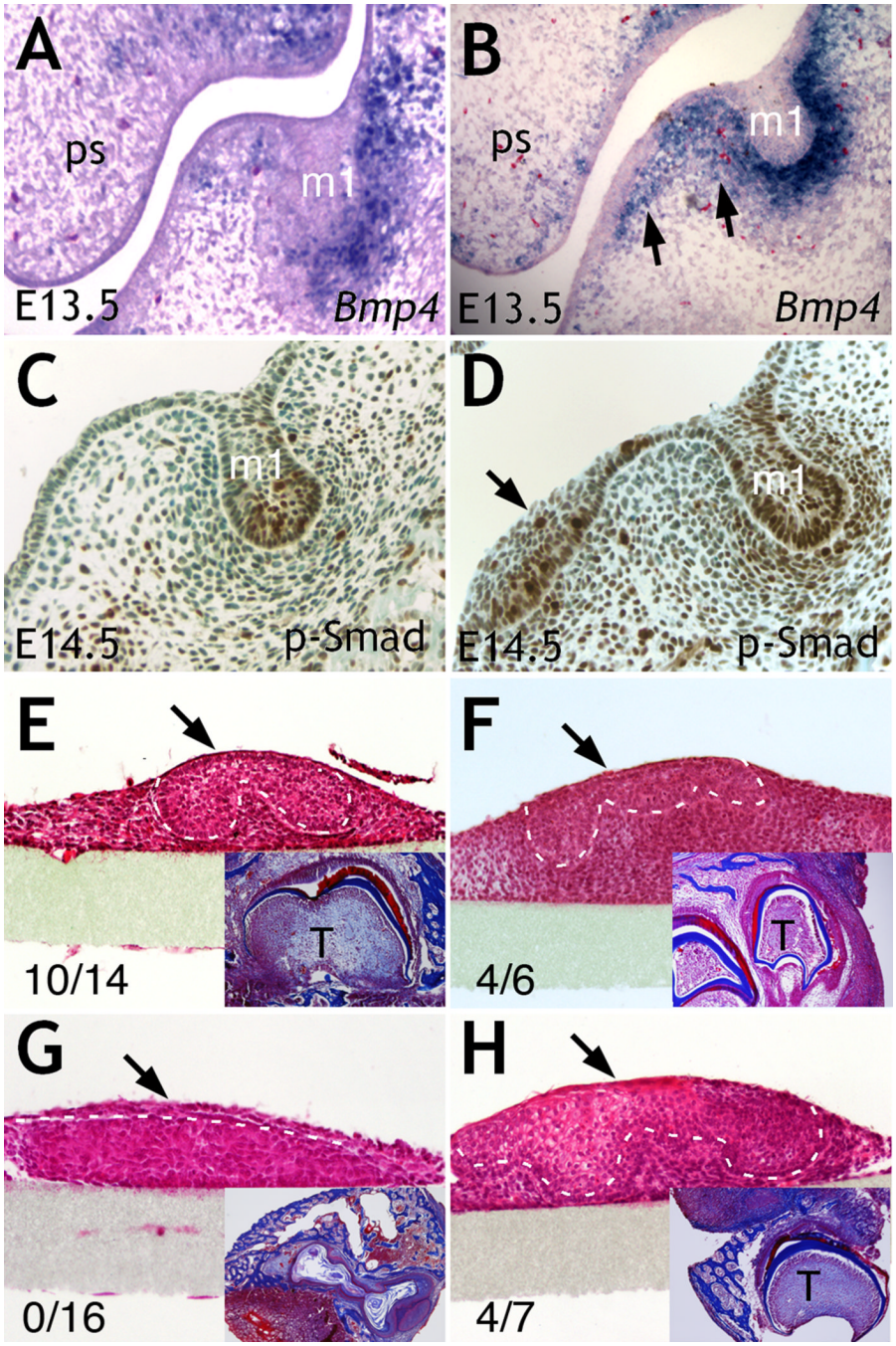

Fig. 3.

(**A, B**) *Bmp4* mRNA expression in E13.5 wildtype (A) and *Osr2*$^{-/-}$ (B) first molar tooth germs. Arrows in B point to lingually expanded *Bmp4* expression. (**C, D**) Increased levels of phospho-Smad1 accompany the supernumerary dental placode (arrow in D) in an E14.5 *Osr2*$^{-/-}$ embryo, compared with wildtype littermate (C). (**E, F**) Isolated molar tooth mesenchyme from E13.5 wildtype (E) and *Osr2*$^{-/-}$ (F) embryos induced tooth formation from E10.5 second branchial arch epithelia. (**G, H**) Isolated mesenchyme lingual to E13.5 molar tooth germ of *Osr2*$^{-/-}$ (H), but not that of wildtype (*G*), induced tooth formation from E10.5 second branchial arch epithelia. White dashed lines in E–H mark the boundary between epithelium and mesenchyme. Insets show sections of renal capsule cultures of corresponding recombinant

explants. Numbers in E–H indicate the ratios of corresponding recombinant explants forming teeth in renal capsules. m1, first molar tooth bud; ps, palatal shelf; T, tooth in renal capsule.

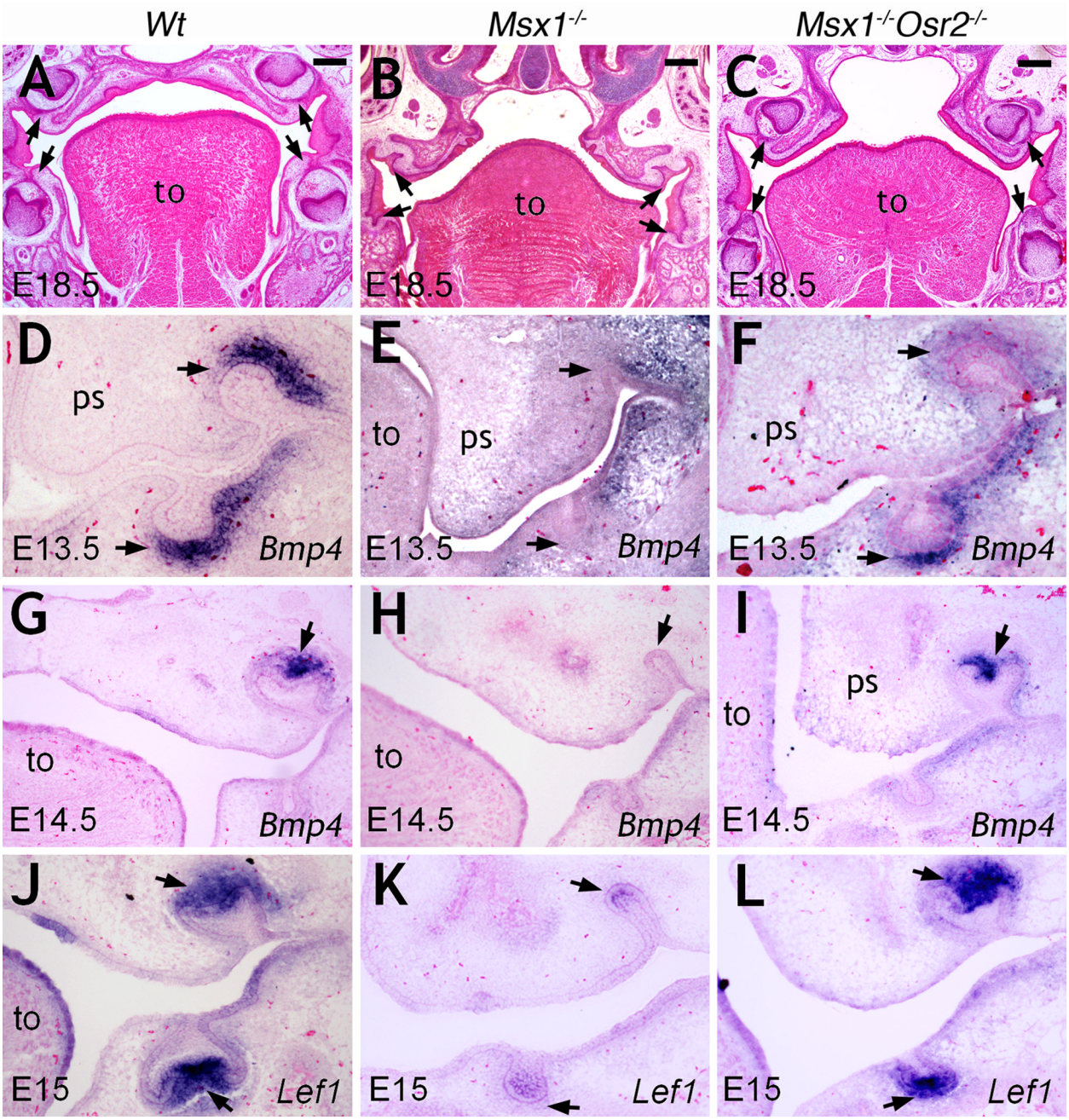

Fig. 4.

(**A–C**) Frontal sections through the first molar tooth germs (arrows) of E18.5 wildtype (A), *Msx*$^{-/-}$ (B), and *Msx1*$^{-/-}$*Osr2*$^{-/-}$ (C) embryos. (**D–I**) *Bmp4* mRNA expression in the first molar tooth mesenchyme (arrows) in wildtype (D, G), *Msx1*$^{-/-}$ (E, H), and *Msx1*$^{-/-}$*Osr2*$^{-/-}$ (F, I) embryos at E13.5 (D–F) and E14.5 (G-I). (**J–L**) *Lef1* mRNA expression in the first molar tooth mesenchyme (arrows) in E15 wildtype (J), *Msx1*$^{-/-}$ (K), and *Msx1*$^{-/-}$*Osr2*$^{-/-}$ (L) littermates. ps, palatal shelf; to, tongue. Scale bar, 200 μm.